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Synthesis of benzofuran derivatives as selective inhibitors of tissue-nonspecific alkaline phosphatase: effects on cell toxicity and osteoblast-induced mineralization

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ABSTRACT

Tissue-nonspecific alkaline phosphatase (TNAP) by hydrolyzing pyrophosphate, an inhibitor of apatite formation, promotes extracellular matrix calcification during bone formation and growth, as well as during ectopic calcification under pathological conditions. TNAP is a target for the treatment of soft tissue pathological ossification. We synthesized a series of benzofuran derivatives. Among these, **SMA14**, displayed TNAP activity better than levamisole. **SMA14** was found to be not toxic at doses of up to 40 μM in osteoblast-like Saos-2 cells and primary osteoblasts. As probed by Alizarin Red staining, this compound inhibited mineral formation in murine primary osteoblast and in osteoblast-like Saos-2 cells.

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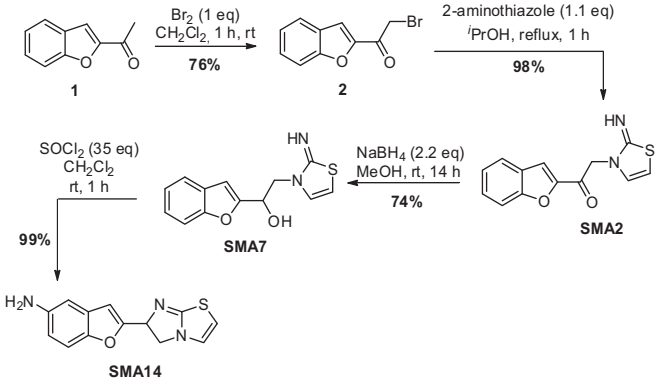
In humans, three of the four alkaline phosphatase isozymes are tissue-specific, the intestinal (IAP), placental (PLAP), and germ cell (GCALP), whereas the fourth is tissue-nonspecific (TNAP) and is expressed in bone, liver, kidney and the central nervous system.[1–3] TNAP hydrolyzes extracellular inorganic pyrophosphate (PP_i), a potent mineralization inhibitor, to enable the physiological deposition of hydroxyapatite (HA).[4] Mineralized cartilage, bone, and teeth are the only tissues in which deposition of calcium phosphate in the form of hydroxyapatite is intended. Ectopic calcification in soft tissues, under pathological conditions, is associated with increased levels of TNAP activity. Therefore TNAP is a therapeutic target for the treatment of soft tissue ossification abnormalities including ankylosis, osteoarthritis, and arterial calcification.[5] Presence of TNAP enriched matrix vesicle in human atherosclerotic lesions was evidenced in vascular calcification.[6] Increase of TNAP activity in arteries was sufficient to remove PP_i secreted by smooth muscle cells and promote vascular calcification.[7]

Several inhibitors were found to be more potent than levamisole.[8–13] Some of them were patented. In this Letter, we provided a series of inhibitors easily synthesizable and more available for exploring the role of TNAP in cells and in tissues. We replaced benzo[*b*]thiophene[13] scaffold by bioisosteric benzo[*b*]furan skeleton. Chemistry could be easily synthesized and adaptable in the already developed conditions.



Scheme 1. Synthetic pathway.

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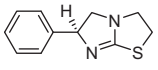
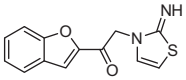
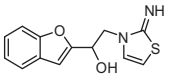
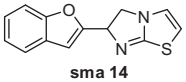
Table 1
Inhibition constants of inhibitors of TNAP, PLAP and IAP

Structure	TNAP			PLAP		IAP	
	IC_{50} (pH 7.8) (μM)	IC_{50} (pH 10.4) (μM)	K_i (pH 7.8) (μM)	IC_{50} (pH 7.8) (μM)	IC_{50} (pH 10.4) (μM)	IC_{50} (pH 7.8) (μM)	IC_{50} (pH 10.4) (μM)
 levamisole	41 ± 1	256 ± 98	47 ± 2	>400	>400	>400	>400
 sma 2	>400	>400	nd	>400	>400	>400	>400
 sma 7	>400	>400	nd	>400	>400	>400	>400
 sma 14	28 ± 2	124 ± 31	32 ± 1	>400	>400	>400	>400

The synthetic chemistry used for the preparation of the benzofuran derivatives is shown in Scheme 1. Bromination of 2-acetylbenzo[*b*]furan in dichloromethane at room temperature provided compound **2** with 76% yield. Subsequent condensation with 2-aminothiazole was performed almost quantitatively. Ketone **SMA2** was reduced classically in the presence of a slight excess of sodium borohydride at room temperature. Upon treatment with thionyl chloride, alcohol **SMA7** furnished cyclized derivative **SMA14**. The overall yield of the synthesis was estimated to 55% for 4 steps (Scheme 1).

The inhibition property of each compound was determined by measuring TNAP activity at alkaline pH (optimum conditions) and at physiological pH. Analog of levamisole having a benzofuran ring was synthesized. This analog and the main synthetic intermediates were evaluated. **SMA14** had better inhibition properties than levamisole (Table 1). Its IC_{50} values either at pH 10.4 or at pH 7.8 were significantly lower as compared with those of levamisole. **SMA-14** behaved in an uncompetitive manner. At 400 μM, **SMA-14** inhibited neither IAP nor PLAP indicating specificity of the inhibition (Table 1).

None of the synthetic intermediates to access **SMA14** was active on any alkaline phosphatase, while **SMA2**, **SMA7** and other displayed IC_{50} over 400 μM.

The cytotoxicity of **SMA14** was tested on human osteoblast like SAOS-2 and MG63, on mouse vascular smooth muscle cell line MOVAS-1 and on mouse primary osteoblast cells, using MTT assay.[14] The cells were incubated without or with inhibitors for 3 days. **SMA14** and levamisole (used as control) were not toxic in the concentration range from 10 to 40 μM on different type of cells (Fig. 1).

We selected osteoblast-like Saos-2 and primary osteoblast cells to analyze the effects of inhibitors on the mineralization. Ascorbic

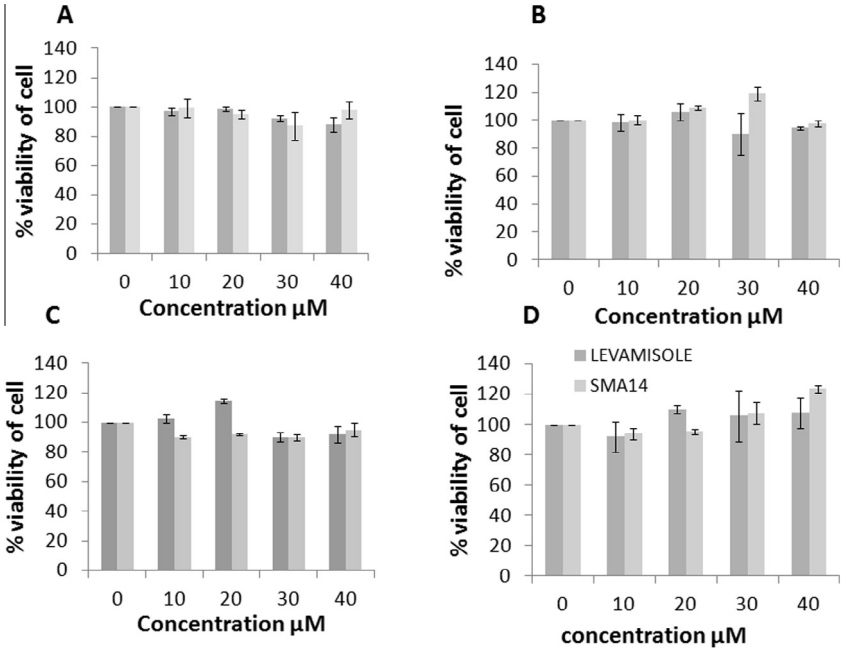


Figure 1. Effect of inhibitors on the viability of (A) mouse primary osteoblast, (B) MG63, (C) SAOS-2, (D) MOVAS measured using MTT assay. Results are expressed as percentage of control containing DMSO 0.1% (mL:mL) without inhibitor. The experiments were repeated in triplicate for each inhibitor ($n = 9$).

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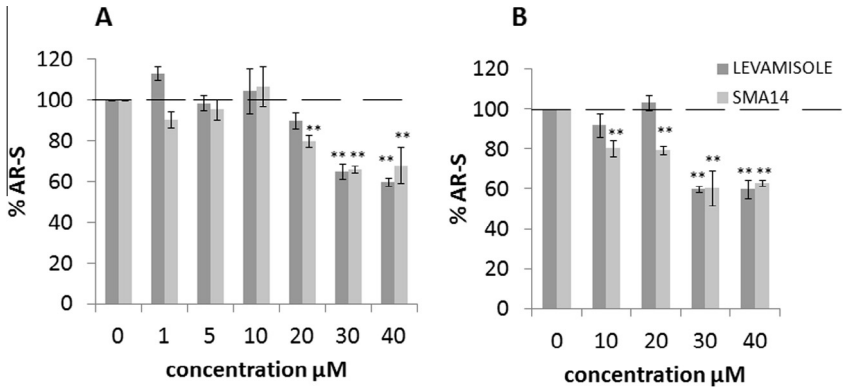

Figure 2. Effect of SMA14 inhibitor on osteoblast-mediated mineralization, induced by (A) Saos-2 cells and (B) primary osteoblasts as monitored by AR-S staining at the indicated concentrations. Results are expressed as percentage of control containing DMSO 0.1% (mL:mL) without inhibitor. The experiments were repeated in triplicate for each inhibitor ($n = 9$).

acid (AA) and β-glycerophosphate (β-GP) served to stimulate osteoblast differentiation and mineralization of Saos-2 cells and primary osteoblast cells.[15,16]

As shown by Alizarin Red-S staining,[17] **SMA14** significantly inhibited mineralization with Saos-2 cells in the concentration from 20 to 40 µM while levamisole decreased it at 30–40 µM to around 40% of control (Fig. 2A). **SMA14** at 10 µM already inhibited mineralization by 20% in primary cells (Fig. 2B) while levamisole did not. Although the effects on mineralization are nearly similar, the solubility property of these two compounds is different. Levamisole is more soluble in water than **SMA14** which may affect tissue selectivity.

In conclusion, we developed a TNAP inhibitor which could be easily synthesized with good inhibition of mineralization induced by osteoblasts and which could be implemented in a drug therapy for ectopic calcification.

Acknowledgments

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Supplementary data

Supplementary data (experimental methods) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.01.061.

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